THE RELATION BETWEEN SOIL AERATION AND THE ACCUMULATION OF ETHANOL AND CERTAIN OTHER METABOLITES IN TOMATO PLANTS

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ABSTRACT

THE RELATION BETWEEN SOIL AERATION AND THE ACCUMULATION OF ETHANOL AND CERTAIN OTHER METABOLITES IN TOMATO PLANTS

by James M. Fulton

The relationship between soil aeration and certain other environmental factors and alcoholic fermentation by Lycopersicum esculentum Mill. was studied. Various degrees of soil oxygen stress were imposed on the roots of the Fireball variety of tomato by varying the soil moisture tension over a narrow range of low values. Ethanol concentration in xylem exudates was taken as a measure of the extent of the fermentation reaction. Ethanol appeared in xylem exudates when the soil supplied less than 38×10^{-8} g oxygen cm^{-2} min^{-1} and the ethanol concentration increased sharply as the supply of oxygen was further reduced. Ethanol accumulation was greater in plants subjected to soil oxygen stress in the light than when subjected to the stress in the dark. Plants bearing open flowers accumulated more ethanol than younger plants. Ethanol was shown to be toxic to tomato plants when added to Hoagland water culture media

at concentrations corresponding to those observed in the xylem exudates.

The effect of soil flooding on the distribution of carbon ^{14}, fixed from $C^{14}O_2$, in alcohol-water extractable metabolites was measured. C^{14} labeled gamma amino butyric acid was observed in the root extracts of flooded plants while this compound was unlabeled in aerobic plants. The total amount was usually greater in plants growing in flooded soils than those in soil with normal moisture regimes. Experiments indicated that gamma amino butyric acid was not toxic.

Low percentages of C^{14} in carbohydrate materials was always associated with high percentages in the lipid fraction. Incorporation of carbohydrate C^{14} into lipid materials was observed under conditions of soil oxygen stress as well as in aerobic soils. It appeared that this reaction taking place under soil oxygen stress might enhance respiration by removing products of the Embden-Myerhof-Parnas pathway, presumably at the level of pyruvic acid.

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By

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LIST OF ABBREVIATIONS

ATP -- Adenosine triphosphate

ADP -- Adenosine diphosphate

DPN -- Oxidized form of diphosphopyridine nucleotide

DPNH -- Reduced form of diphosphopyridine nucleotide

TPN -- Oxidized form of triphosphopyridine nucleotide

O.D.R. -- Oxygen diffusion rate

M -- Molar

L.S.D. -- Least significant difference

INTRODUCTION

A primary function of oxygen in the biological system is to serve as a hydrogen acceptor in respiration. Through the process of photosynthesis, green plants transform a portion of the incident solar energy into chemical energy which is stored in the form of carbohydrates. Subsequent transformation of the carbon fixed by photosynthesis into tissue compounds necessary for growth requires expenditure of energy to drive many of the reactions in the desired direction. These latter reactions are not photochemical in nature so that solar energy cannot be used directly but indirectly by release of the chemical energy previously stored in carbohydrates. In the biological system oxidation of the carbohydrates consists of a transfer of hydrogen atoms to the phosphopyridine nucleotides and ultimately through the cytochrome system to molecular oxygen. Cells of plant roots are dependent upon oxygen of the soil atmosphere for this purpose (32).

The oxygen content of the soil atmosphere is dependent upon a capacity factor related to the proportion of the total soil volume occupied by voids and an intensity factor

represented by the composition of the gaseous phase. The void volume or aeration pore space is fixed by the bulk density of the soil which may in turn be modified by cropping systems, cultivation, incorporation of organic matter and the moisture content of the soil. The partial pressure of oxygen in the soil atmosphere is determined by the difference between the rate of utilization and replenishment from the above ground atmosphere. The flux of oxygen from the above ground atmosphere to the soil pores is largely a diffusion process which has been the subject of numerous investigations many of which have been reviewed by Wesserling et al (50).

The movement of oxygen, from the soil atmosphere to the living root, is also a diffusion process and depends on the concentration gradient between the gaseous phase and the root itself (24, 26, 36). The rate of oxygen diffusion is likely to be the limiting factor because the root is commonly bathed by a moisture film and diffusion through liquid is of the order of 10^4 times slower than in air (41). Lemon (26) shows from theoretical considerations that when the oxygen concentration at the root surface is low, diffusion controls the rate of oxygen uptake. Thus at a given oxygen concentration in the soil atmosphere and for any given oxygen demand by the root, the thickness of the moisture

film surrounding the root determines the "oxygen supplying power" of the soil. This fact gains additional significance if we consider the situation where a plant has been established in a given soil. Specifying the soil arbitrarily fixes most of the soil properties which influence aeration. The outstanding exception is the soil moisture content which varies with precipitation, evaporation and transpiration. Immediately following periods of high precipitation, the voids are inaccessible to air because they are largely filled with water. Subsequently an increasing proportion of the total soil pores become accessible to air as water is withdrawn from the larger pores by drainage. In addition, the increased soil moisture tension concomitant with lower water tables reduces the thickness of the moisture film surrounding the soil particles and plant roots. Still smaller pores are drained and thinner films are produced by absorption of water into the plant and its elimination into the atmosphere by transpiration.

Because plant growth or yield reflects the integrated effects of a great many biochemical processes, it would be desirable to learn what specific reactions are influenced by oxygen stress and to learn as much as possible about the nature of the relationship between such specific reactions and

soil aeration.

One method of determining which reactions are influenced by aeration is to subject plants to both aerobic and anaerobic soil conditions and to analyze the plant tissue for as many metabolic intermediates as possible. From differences in the concentration of metabolites caused by anaerobiosis and existing knowledge of plant biochemistry, it may be possible to postulate which reactions are being affected. Furthermore the presence or absence or any marked change in the concentration of a metabolite prompted by anaerobic conditions might suggest an assay technique which would reflect oxygen stress in the plant.

The first part of this study was undertaken to determine the relationship between soil oxygen stress and the production of ethyl alcohol. It was reasoned that if a sufficiently sensitive assay for ethyl alcohol in plant tissue could be developed it would be possible to study the relationship between soil oxygen stress and ethanol accumulation by plants. Further aims of this research were to measure the magnitude of soil oxygen stress necessary to promote alcoholic fermentation in plants, to study the nature of the relationship between soil oxygen stress and ethanol accumulation and to search for other metabolic

intermediates whose presence or concentration are influenced by soil aeration.

LITERATURE REVIEW

Development of the platinum micro-electrode technique for measuring oxygen diffusion rates by Lemon and Erickson (25) provided a means of characterizing the oxygen supplying power of soil and prompted a series of investigations concerning the relationship between soil oxygen availability and plant growth (10, 12, 27, 28, 42, 51). These studies have shown that oxygen stress periods as short as one day out of the whole growing season will reduce total growth, that sensitivity to oxygen stress varies with stages of growth (10, 47) as well as with plant species.

As in other tissues, oxygen participates in plant respiration only as a final acceptor of electrons (5). The respiratory substrates and intermediates do not themselves react with oxygen, but the electrons removed during oxidation of substrates are transferred by way of a series of carriers which are capable of yielding electrons to oxygen with the formation of water. It is now certain that the Embden-Myerhof-Parnas sequence of reactions (5, 13, 18) are important in the aerobic breakdown of carbohydrates in plant tissues. Other reaction sequences, particularly the

pentose phosphate pathway, no doubt make a significant contribution under certain conditions but the Embden-Myerhof-Parnas sequence of reactions is commonly considered the most important pathway from glucose to pyruvic acid in air (5).

$$\text{Glucose} \longrightarrow 2\ \text{Pyruvic acid}$$

In air, pyruvate is oxidized further through the reactions of the tricarboxylic acid cycle where the bulk of the energy useful to the cell is produced.

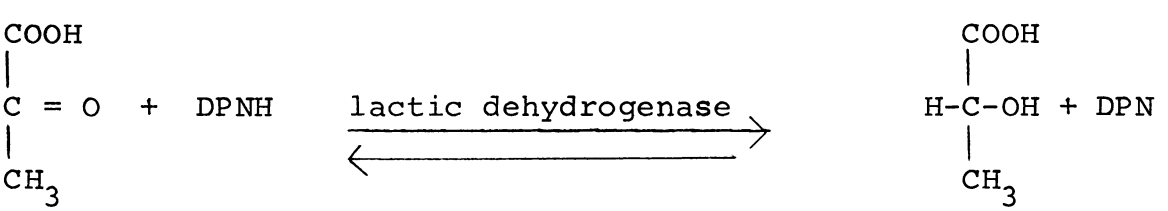
Normally all of the pyruvate produced is oxidized, however under some conditions, the rate of pyruvate formation exceeds its oxidative removal. One means of reducing pyruvate oxidation is to restrict the oxygen supply (5). In these circumstances the pyruvate may be decarboxylated to acetaldehyde by pyruvate decarboxylase and if reduced diphosphopyridine nucleotides (DPNH) are present the acetaldehyde is reduced to ethanol by alcohol dehydrogenase.

$$\begin{array}{l} \text{COOH} \\ | \\ \text{C} = \text{O} \\ | \\ \text{CH}_3 \end{array} \xrightarrow{\text{pyruvic decarboxylase}} \begin{array}{l} \text{CHO} \\ | \\ \text{CH}_3 \end{array} + \text{CO}_2$$

$$\begin{array}{l} \text{CHO} \\ | \\ \text{CH}_3 \end{array} + \text{DPNH} \underset{\longleftarrow}{\xrightarrow{\text{alcohol dehydrogenase}}} \begin{array}{l} \text{CH}_2\text{OH} \\ | \\ \text{CH}_3 \end{array} + \text{DPN}$$

Restricted oxygen supply increases the amount of DPNH present by preventing terminal transfer of electrons to oxygen. Both enzymes necessary for conversion of pyruvate to ethanol are widely distributed in plant tissues (5, 8, 41) so that the appearance of ethanol under reduced oxygen supply is readily explained.

Several reports (33, 39, 48) show an accumulation of alcohol by plant tissues under anaerobic conditions. Kenefick (22) working with intact sugar beets showed an accumulation of ethanol in the storage tissues of the root when the plant was placed in a nitrogen atmosphere and that when the plant was subsequently returned to a normal atmosphere the alcohol disappeared. He also showed that at least a portion of the accumulated alcohol was dissipated in the form of ethanol vapor from the foliage. Less ethanol accumulated when the anaerobic conditions were confined to the root zone. Occasionally plant tissues contain the lactic acid dehydrogenase enzyme and lactic acid accumulates during oxygen stress.



The initial process in the tricarboxylic acid cycle involves decarboxylation of pyruvic acid to acetaldehyde which subsequently condenses with oxalacetic acid to form citric acid. A succeeding series of reactions involves four dehydrogenations and two decarboxylations. In this way the three carbons of pyruvic acid are oxidized to carbon dioxide and five nucleotide molecules are reduced.

Elimination of oxygen from the system prevents the reoxidation of the reduced nucleotides resulting in a shortage of the oxidized forms and preventing operation of the cycle. In addition to pyruvate, the cycle serves as a pathway for the final breakdown of two carbon units from fats and proteins. Furthermore the enzyme L-glutamic dehydrogenase catalyzes the reaction between α ketoglutarate and ammonia giving rise to glutamic acid. Transamination reactions between glutamic and the α keto acids produce the appropriate amino acids (13). Thus, through its effect on the oxidation of reduced pyridine nucleotides and the Embden-Myerhof and tricarboxylic acid sequence of reactions, oxygen stress could theoretically influence a large portion of the total plant chemistry.

MATERIALS AND METHODS

Fireball tomatoes, Lycopersicum esculentum Mill., were used almost exclusively throughout these experiments and all references to plants or plant materials refers to this species unless specifically stated otherwise. Several of the experiments conducted were suggested from examination of results obtained from preceding experiments so that interpretation was simplified by considering data from certain groups of experiments collectively. For this reason the report has been prepared according to the subject matter sequence given in the table of contents.

Soil Properties

The soil was a "greenhouse potting mixture" used for propagation of potted plants in the soil science greenhouse. Triplicate three-inch core samples were collected from pots in which experimental plants had been grown. Bulk density and the volume of pores drained at various tensions on a Leamer and Shaw (23) type tension table were measured. The average bulk density of the soil was 1.21 grams per cubic centimeter as measured with the triplicate

three-inch core samples. Assuming the specific gravity of the soil material to be 2.65, substitution in the following equation (3) showed that 54.5 percent of the total volume of oven dry soil was occupied by air.

$$\text{Total air pore space (\%)} = 1 - \frac{\text{Bulk density}}{\text{Specific gravity}} \quad X\ 100$$

The volume of air filled pores was calculated for each water table position from the weight lost by the soil core, as the water table was lowered from the surface to 2.5, 5.0, and 7.5 cm and from the dimensions of the pots used. These data are presented in Table 1.

Table 1. Calculated air capacity of potting soil in six inch pots at various water table positions.

Depth to water table	Volume of soil above water table	Volume of air-filled pores
cm	cc	cc
0	0	0
2.5	384	20
5.0	729	48
7.5	1092	102

With this soil, small changes in the water table position or moisture tension produced appreciable changes in the volume of air filled pores.

Ethanol Assay

An ethyl alcohol assay was developed by preparing a series of standard aqueous solutions of ethanol containing 0.37, 3,7, 7.4, 18.5, 37, 74, 185 and 370 parts per million by weight. Samples, 20 µl in volume, were injected into the Beckman GC2A gas chromatograph equipped with a hydrogen flame detector and a four foot partition column containing 8.5 grams chromosorb W as support material, 1.7 grams of diethylene glycol succinate and 0.3 grams of phosphoric acid as the active agent. The fuel for the flame was hydrogen and air with helium as the carrier gas at a gauge pressure of 30 pounds per square inch giving a flow rate of approximately 82 milliliters per minute. The ethyl alcohol was eluted from the column 1.8 minutes after injection, the carbon atoms were ionized by the hydrogen flame and the resulting ion current recorded using a Sargent model SR recorder equipped with a one millivolt range plug and a Disc integrator. The area under the peak traced by the recorder varied with the number of carbon atoms passing the detector so that a standard curve was obtained by plotting alcohol concentration in ppm versus peak area. The curve was frequently reproduced from freshly prepared alcohol standards to ensure that instrumental sensitivity had not

changed.

Samples of methyl, n-propyl, isopropyl and butyl alcohol were subjected to chromatography under conditions identical to those used in preparing the ethyl alcohol standard curve. The elution times were as follows: Methyl alcohol 1.6 minutes, n-propyl 2.4 minutes, isopropyl 1.7 minutes and butyl alcohol 5.6 minutes. The presence of methyl or isopropyl alcohol in a sample of unknown composition would interfere with the quantitative estimation of ethyl alcohol; however their presence would be detected by the appearance of multiple peaks near the 1.8 minute elution time. Better resolution was obtained at lower column temperatures but the elution peaks were less symmetrical making quantitative measurements more difficult. The possible number of compounds which could be extracted from plant tissues and which would interfere with the detection of alcohol are unknown. All that can be said with certainty is that peaks appearing at 1.8 minutes represent a compound which chromatographs identically with and appears to be ethyl alcohol. Throughout the remainder of this report such peaks are assumed to be ethyl alcohol. Known samples were frequently chromatographed before and after unknowns and in all cases the elution peaks appeared to be identical.

Figure 1 shows the symmetry of the elution peaks obtained on chromatography of aqueous ethanol solutions obtained during preparation of a duplicate standard curve one month after preparation of the original. The attenuator settings indicate the amount of signal reduction necessary to keep the peaks obtained at the three concentrations on scale. The actual concentrations were calculated from the weight of ethanol and water used in preparing the duplicate standards and the observed values were obtained by measuring the area under the peaks and reading the corresponding ethanol concentration off the original standard curve shown in Figure 2. The reproducibility obtained was adequate to compare the rather large differences in alcohol concentration obtained in subsequent experiments.

Description of Light Chamber

During preliminary attempts to measure ethyl alcohol in xylem exudates of flooded tomato plants growing in the greenhouse, it was observed that the alcohol concentration appeared to vary with the intensity of the incident sunlight. In order to remove this variable a light chamber similar to that described by Rawlins and Moss (35) was constructed (Fig. 3) and placed in the laboratory. Subsequently all

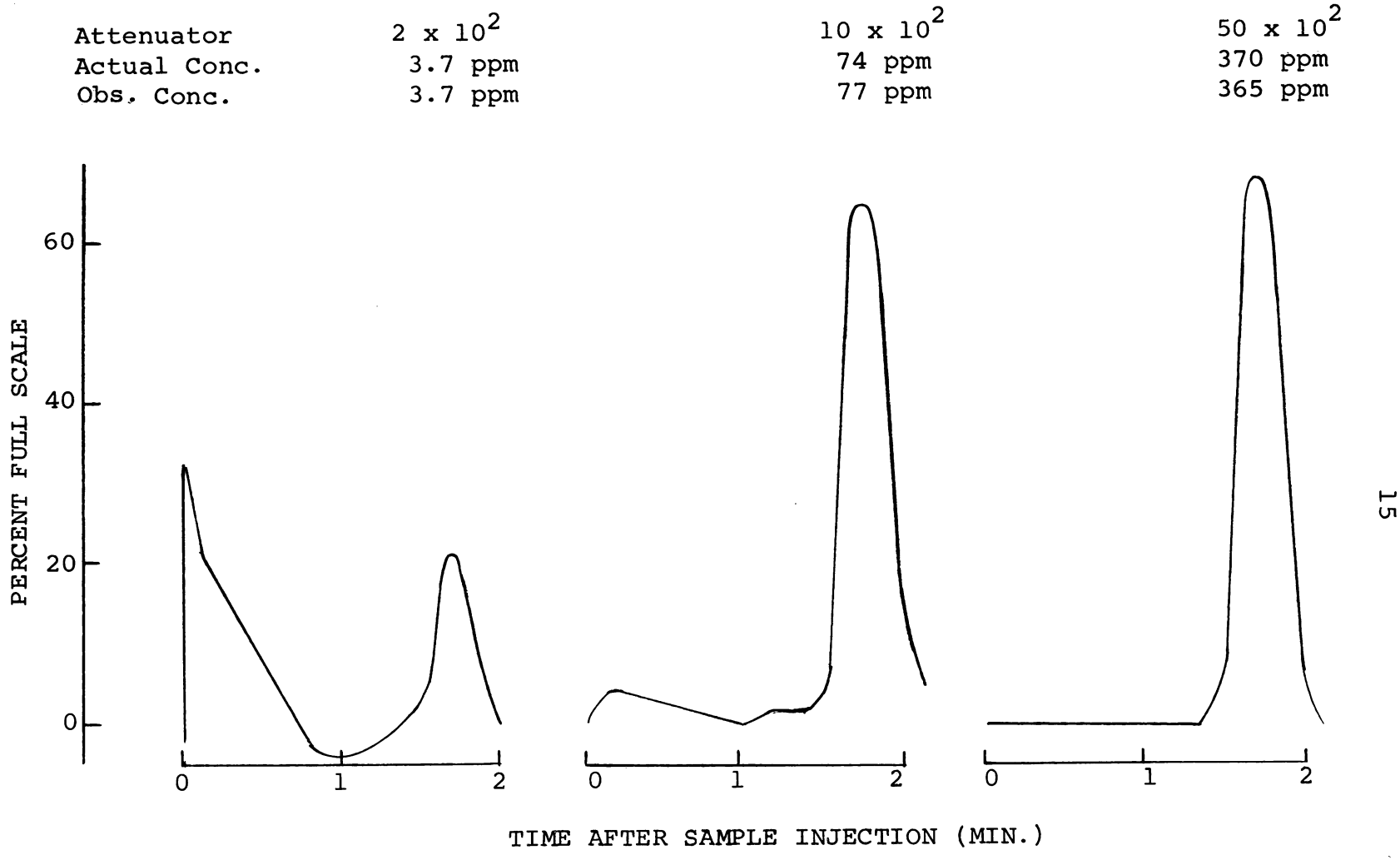


Figure 1. Ethyl alcohol elution peaks from the Beckman GC2A gas chromatograph with hydrogen flame detector.

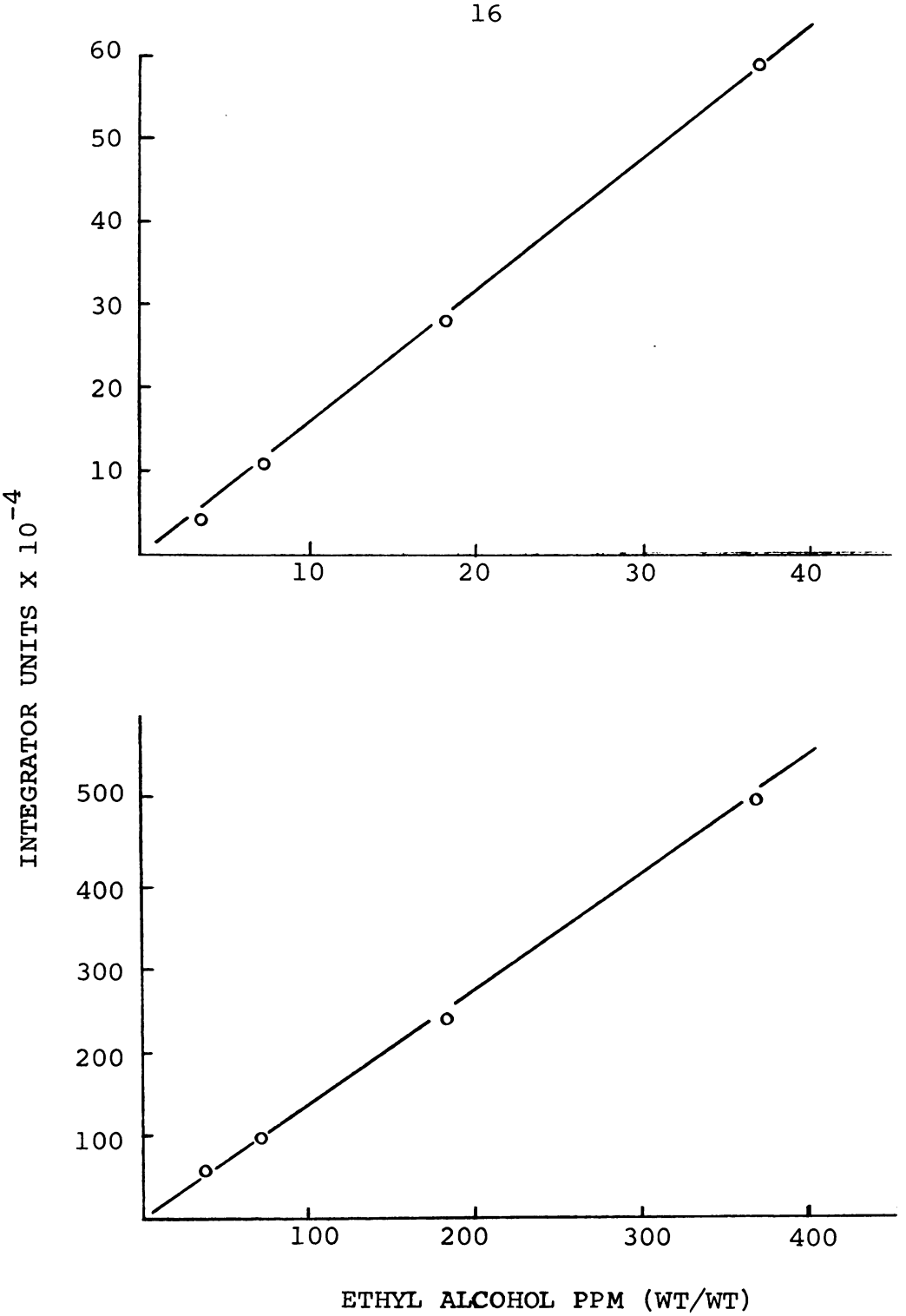


Figure 2. Standard ethyl alcohol curves obtained with a Beckman GC2A gas chromatograph with hydrogen flame detector.

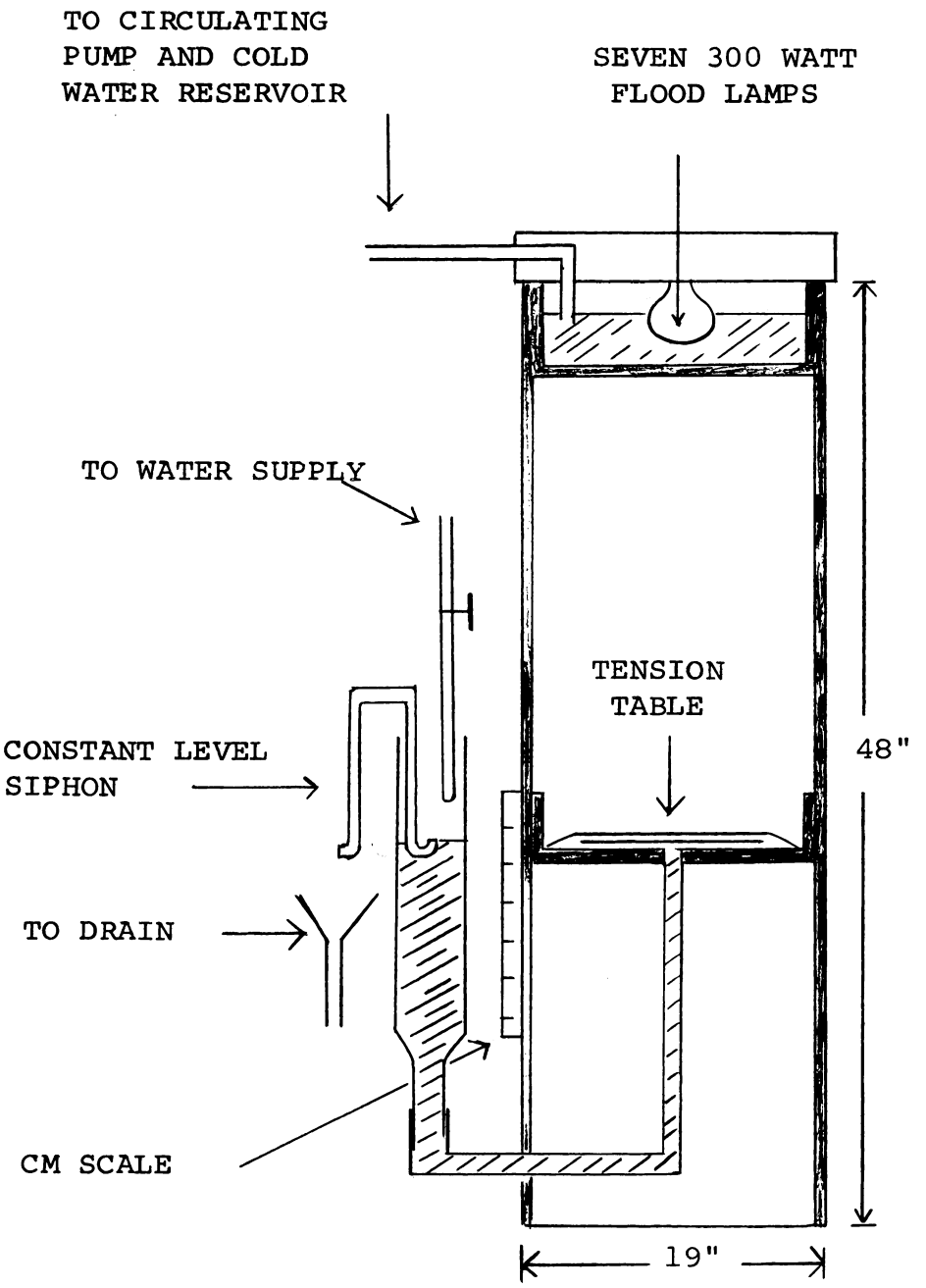


Figure 3. Light chamber with tension table installed.

plants were grown in six inch clay pots in the greenhouse until they reached the stage of growth desired in the experiment concerned and were then moved to the laboratory during the experimental period. The chamber provided a light intensity of 9000 foot candles at the source, about 6000 foot candles at the uppermost leaves and about 4000 foot candles midway between the soil surface and the uppermost leaves.

Cool water, circulated through the light tank, reduced heat production and filtered out some of the infrared radiation. Soil placed on the tension table was subjected to desired moisture tensions by adjusting the position of the constant level siphon. Tap water added dropwise replaced water used by the plants. The low tensions used in these experiments were maintained within one millimeter.

Statistical Analyses

Experimental variability was evaluated by means of analyses of variance. Each of the experiments except those testing the effect of light intensity and the one with shallow rooted plants were designed as randomized block experiments. In the randomized block experiments, variation due to treatment, replication and experimental error was evaluated. The light intensity and experiments with shallow

rooted tomatoes were set up as completely randomized experiments and the variability among plants treated alike was used to test the significance of the difference between plants treated differently. A logarithmic conversion was employed in experiments studying the time course of ethanol accumulation where the very small values associated with one treatment were compared with the rather large values associated with other treatments.

Effect of Soil Flooding on the Distribution of Carbon^{14} Experiments

In each experiment, four plants enclosed in a glass photosynthesis chamber on the greenhouse bench were supplied approximately one half a millicurie of $C^{14}O_2$ by reacting the appropriate quantity of Ba $C^{14}O_3$ with an excess of 2N lactic acid. The chamber remained closed for two hours during which time it was turned frequently to ensure uniform exposure to sunlight. The potted plants were then removed from the chamber and placed on the greenhouse bench. Subsequently, one half of the pots were flooded to the soil surface for varying periods of time while the remaining half were subjected to normal watering procedures and served as controls for each of the flooding treatments. Leaves, stems and roots of all plants were harvested separately at

the end of the flooding period and boiled in 80% ethanol for ten minutes. The alcohol was decanted, about fifty ml of water added and boiling continued for about five minutes. The alcohol-water extracts were combined in 250 ml graduates, allowed to cool and the volume recorded. To one half ml of each extract on glass planchets, one drop of 2N acetic acid was added. The samples were evaporated to dryness under a heat lamp and the activity of the C^{14} counted using an end-window counter. Approximately one third of the remaining alcohol-water extract was reduced at 45°C under vacuum to a volume of about 50 µl. Approximately 25 µl were transferred to the origin of a Whatman #1, 18 x 22 inch, chromatographic paper and developed in one direction with water saturated phenol and in the second direction with butanol propionic acid (6).

Spots bearing labeled carbon were located by placing the chromatogram against Kodak no-screen x-ray film in the dark for about three weeks before development. The activity of C^{14} in each spot was measured by counting with a GM chromatogram tube. The amount of C^{14} in each spot was expressed as percent of the total on the chromatogram.

All but one of the chromatograms were sprayed with a solution composed of 200 mg ninhydrin, 90 ml of 95%

ethanol, 5 ml of 2,6-lutidine and 5 ml of water in order to assess the relative amounts of total amino acids (labeled plus non labeled). The chromatograms were dried at 90°C for two minutes and the intensity of ninhydrin positive spots rated as follows: 0 - no color observed, 1 - faint spot detectable and 2, 3, 4 and 5 representing progressively darker or more intense color development. The alanine, glutamine and γ amino butyric acid spots from the one chromatogram which was not sprayed with ninhydrin were eluted with water and cochromatographed with non labeled known materials. The remaining compounds on all chromatograms were identified by comparing with chromatograms previously prepared by Tolbert (15, 34, 44, 45) of the Plant Biochemistry Laboratory at Michigan State University, using techniques described by Benson et al (6).

EXPERIMENTAL

Soil Aeration and Plant Growth

Description of Experiments

The susceptibility of the plants to short periods of oxygen stress was evaluated by flooding the soil with water for a period of twenty-four hours once during the period from emergence to maturity. Seven plants, each constituting a replication, were flooded at 3, 4, 5, 6, 7, 8 or 9 weeks post emergence. The fresh weight of fruit and the dry weight of the foliage were recorded as a measure of treatment effect. The effect of continued oxygen stress over a period of two weeks was evaluated by comparing the dry and fresh weight of plants watered in the normal fashion with plants grown with the water table at (a) the surface, (b) 2.5 cm below the surface, and (c) 10 cm below the soil surface.

Results and Discussion

The dry weight yield of tomato foliage was reduced by flooding the soil for a period of 24 hours when the plants were three, four or five weeks old (Table 2).

Table 2. Effect of a twenty-four-hour oxygen stress, applied at different stages of growth, on yield of tomatoes harvested when fruit was ripe.

Date flooded	Age weeks	Dry weight foliage g/pot	Fresh weight fruit g/pot	O.D.R.* March 16
Feb. 12	3	22.5	732.1	38.9
18	4	23.0	716.9	36.6
26	5	27.7	797.7	31.3
Mar. 7	6	29.2	851.7	29.3
14	7	31.9	929.9	35.3
18	8	33.0	941.7	30.7
25	9	30.3	826.0	35.7
No treatment		32.1	873.4	----
L.S.D. P. 05		3.5	120.7	

*O.D.R. = oxygen diffusion rate in g x 10^{8} cm^{-2} min^{-1}

The younger plants were more susceptible than the more mature ones. Plants flooded either three or four weeks post emergence produced a small yield of fruit. Van Doren (47) reported similar results for a similar experiment except that the yield of fruit was not influenced by short term flooding treatments. The oxygen diffusion data included in Table 2 show that the aeration condition of the soil, subsequent to flooding, was at least equal to that in soil which had not been flooded. The significant feature, of

experiments of this type, is the evidence that short periods of oxygen stress imposed by flooding at an early age restricted plant growth. Although the nature of the injury is as yet unknown, the damage was sufficiently permanent that final yield was depressed. Reduced production of high energy phosphate bonds associated with oxygen stress would not seem to be a sufficient explanation because this situation would be corrected at the conclusion of the stress period.

Data in Table 3 show that the magnitude of the yield reduction depended upon the severity of the flooding conditions. However, soils flooded to the surface or 2.5 cm below the surface produced equal yields. There is no evidence that the dry matter percentage was influenced by the flooding treatments. The yield reduction observed under continuous stress could be associated with the inhibition of oxidative phosphorylation and subsequent reactions involving adenosine triphosphate. Comparison of data in Tables 2 and 3 indicate a greater proportionate yield reduction under continuous than short term stress. It should be pointed out that plants used in obtaining the continued stress data were not grown to maturity. Plants subjected to short term stress were grown to maturity and may have regained some of the loss subsequent to flooding.

Table 3. Effect of varying degrees of oxygen stress on growth of young tomato plants (harvested five weeks post emergence).

Water table position	Green weight g/pot	Dry weight g/pot	Dry matter %
Surface	13.9	1.1	7.6
-2.50 cm	14.5	1.4	9.8
-10.0 cm	48.7	3.8	7.9
Normal watering	89.5	5.8	6.4
L.S.D. P. 05	12.7	1.2	--

Soil Aeration and Ethanol Accumulation

Plants Bearing Flowers

Description of Experiments: Variations in soil oxygen stress were obtained by flooding plants bearing three open flowers and growing in six inch clay pots so that the water table was (a) at the surface of the soil, (b) 2.5 centimeters below the surface and (c) 6.25 centimeters below the surface. Each aeration treatment was conducted in quadruplicate. Immediately after flooding the plants were "decapitated" by severing the hypocotyl with a sharp knife after which short pieces of rubber tubing were attached to the protruding stub. Exudates accumulated in sufficient quantity to permit alcohol determinations within fifteen

minutes of flooding. Subsequent samples were collected at two, four, eight, twelve and twenty-four hours after flooding.

Results: Figure 4 shows that alcohol concentration increased progressively with time of treatment reaching a maximum at approximately twelve hours. The maximum attained was 422, 179 and 16.7 ppm in plants flooded to the surface, 2.5 and 6.25 centimeters below the surface respectively. The large change in alcohol concentration effected by relatively small changes in the water table position provided evidence that small changes in the moisture and aeration relationships of a soil can produce striking changes in the metabolic processes occurring in the plant root. Each point on these curves represents the average from four different plants and although considerable plant to plant variability existed as shown in Table A of the Appendix, the analyses of variance indicated that statistically significant differences existed at all points in excess of four hours of treatment.

Pre-bloom Plants

Description of Experiment: Although the plants used in the previous experiment were selected so that all carried three open flowers at time of flooding, it was impossible to obtain all plants at exactly the same stage of development

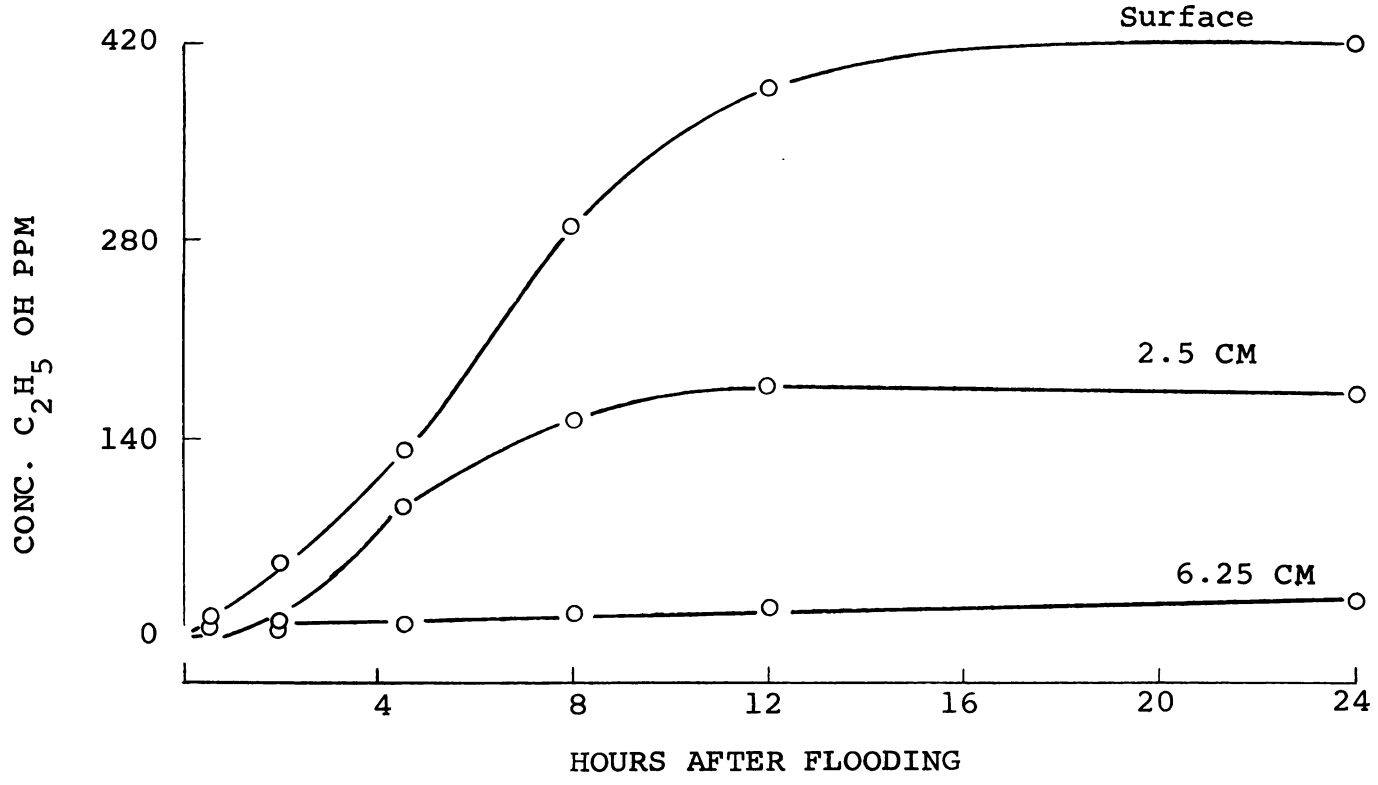


Figure 4. Time course of ethanol accumulation in xylem exudates of flowering tomato plants subjected to water tables at the surface of the soil, 2.50 and 6.25 cm below the surface. (Each point the average of four determinations.)

and upon analyses of the samples it appeared that some of the plant to plant variability in alcohol concentration was associated with stage of growth. In order to obtain a measure of this effect the above experiments were repeated using plants bearing buds but no open flowers.

Results: Figure 5 shows the data obtained for the same flooding treatments applied to pre-bloom plants. The mean ethanol concentration reached a maximum about twelve hours after treatment and the maximum increased sharply with the severity of flooding. However, the outstanding feature was that the maximum reached by plants in bloom was almost four times greater than plants bearing buds only. This study provided no clues to the reasons for greater alcohol production by plants bearing flowers; however, increased respiration rates associated with meristematic tissue (32) may be a factor. Initiation of the fruiting process may provide sufficient new meristematic tissue to cause an increased utilization of carbohydrates which under conditions of oxygen stress are diverted to alcohol.

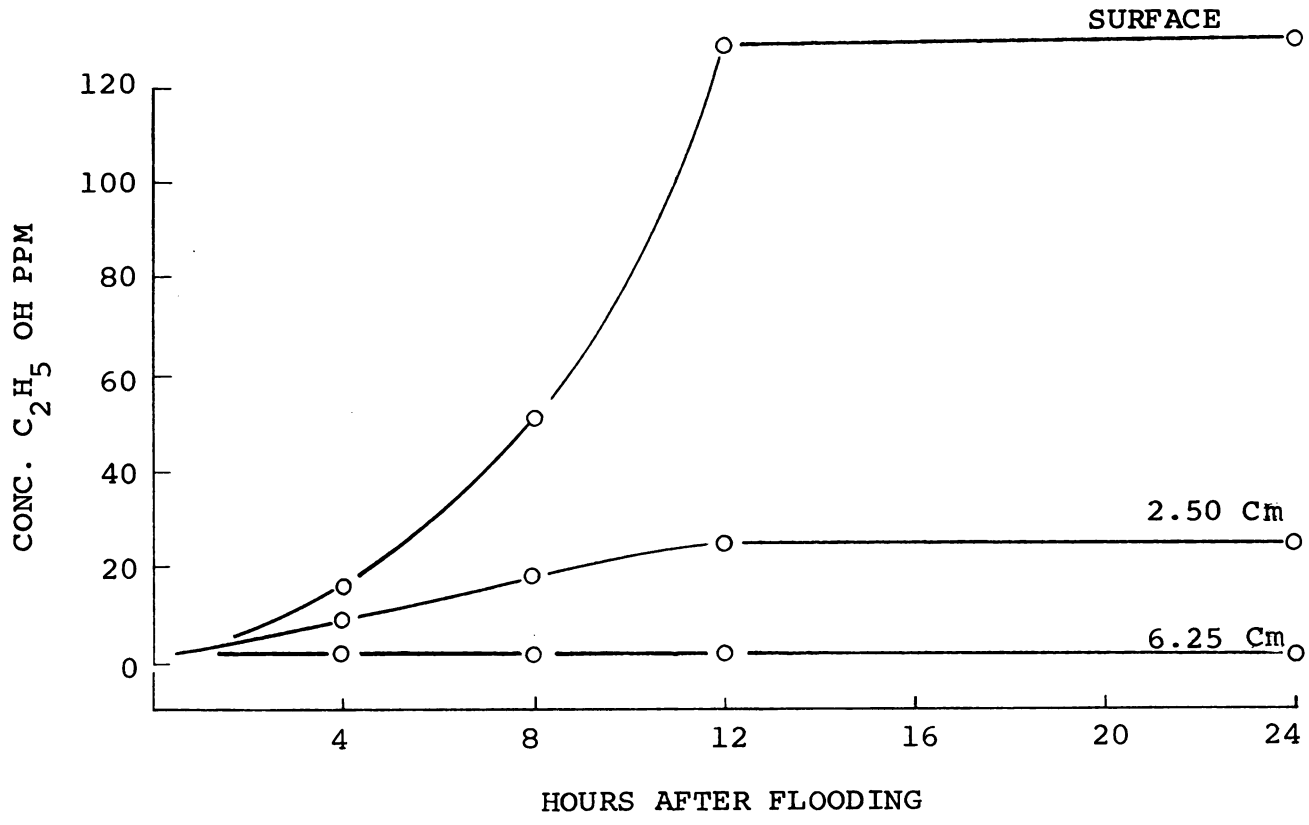


Figure 5. Time course of ethanol accumulation in xylem exudates of pre bloom tomato plants with water tables at the surface of the soil, 2.50 and 6.25 cm below the surface. (Each point represents the average of three determinations.)

Effect of Decapitation

Description of Experiment: Because Kenefick (22) had shown that the foliage of sugar beet plants possess a mechanism for dissipation of ethanol, it was considered necessary to measure the effect of "decapitation" at the time of treatment on the alcohol concentration of the exudate samples. For this purpose, plants bearing three open flowers were brought to the laboratory, placed in the light chamber and the soil flooded to the surface with water. However, in this case, the plants were not decapitated until immediately prior to sampling at four, eight, twelve and twenty-four hours after flooding. In subsequent discussion these plants will be referred to as "intact plants" denoting that the foliage was not removed until time of sampling. These intact plants were subjected to light during the first twelve hours of treatment and to darkness from the 12th to the 24th hour.

Results: Removal of foliage at the time the soil was flooded (decapitated plants) resulted in a higher alcohol concentration than in plants left intact until sampling time. Figure 6 shows 392 ppm of ethanol in the xylem exudates of decapitated plants compared to 238 ppm for intact plants twelve hours after flooding. This

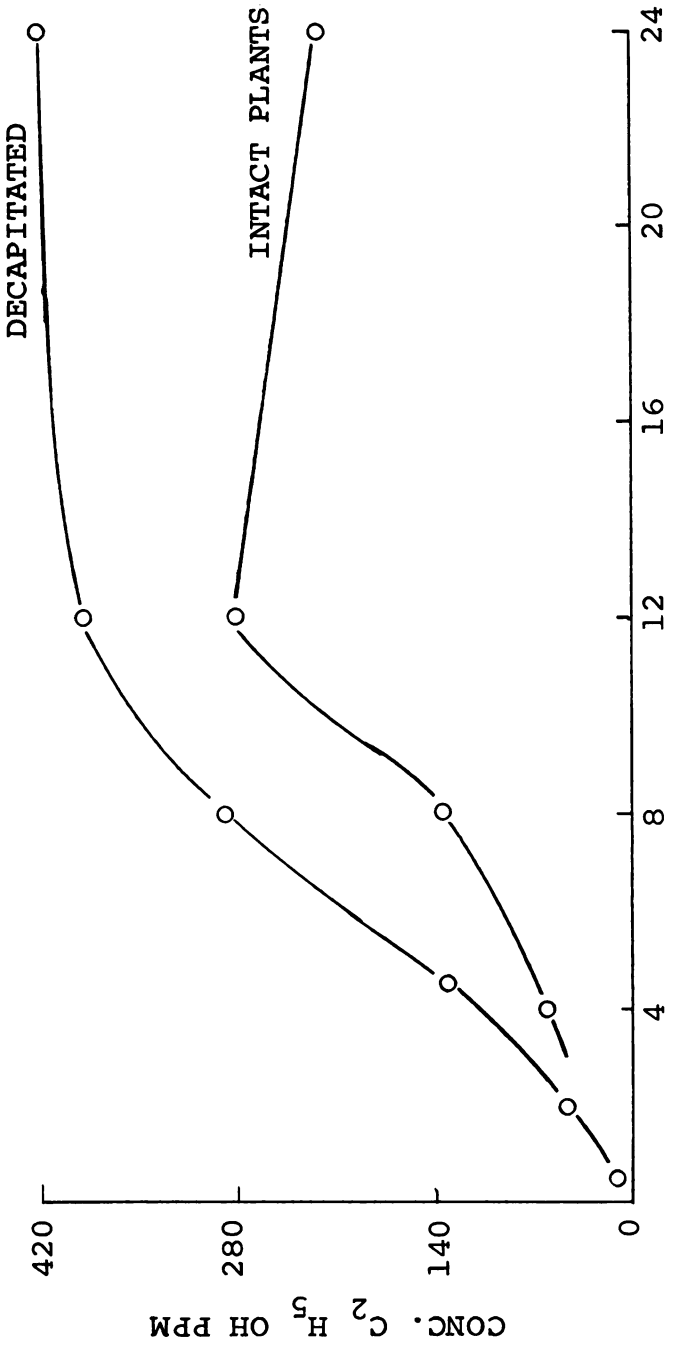


Figure 6. Dissipation of ethanol by foliage of flowering tomato plants flooded to the surface of the soil.

difference was probably due to elimination of ethanol by the foliage in the intact plants. Kenfick (22) described such a mechanism operative in intact sugar beets. The apparent decrease in alcohol content of samples collected from intact plants after 24 hours of flooding compared to an apparent slight increase for the "decapitated" plants may reflect the effects of light intensity. It will be recalled that the lights were turned on during the first 12 hours and off during the 12th to the 24th hour. Experiments described later were designed specifically to measure the light effect and tend to support the above conclusions. Each point shown in Figure 6 represents the average of four determinations for the decapitated plants and three for the intact plants. However, the data were obtained in two separate experiments so that statistical comparison was not justified.

Intact Plants

Description of Experiment: Having established from the previous experiments that (a) the ethanol concentration of the xylem exudates approached a maximum twelve hours after flooding, (b) the maximum ethanol concentration obtained was greater in plants bearing flowers than pre-bloom and (c) the ethanol concentration of intact plants was less than

in decapitated plants it seemed desirable to incorporate these factors into experiments designed to measure the effect varying degrees of soil aeration on the ethanol concentration of xylem exudates.

Variation in soil aeration was achieved as before by flooding the soil to the surface, 2.5 and 6.25 cm below the surface. The potted plants bearing three open flowers were set into plastic pails, placed in the light chamber and water was added until it reached the appropriate depth. The lights in the chamber were turned on during the whole twelve hour experimental period. Because the light chamber was not large enough to hold all plants in the experiment at one time, each of the four replications was conducted on a separate day.

The effect of the three flooding treatments on soil aeration was characterized by measuring the oxygen diffusion rate two centimeters below the soil surface with the platinum micro electrode (25). Ten electrodes were placed in each pot ten hours after flooding and the diffusion current measured five minutes after applying a potential of 0.65 volts.

Results: Table 4 reports the ethanol concentration produced at three levels of soil aeration achieved by using

three water table positions. The very small change in oxygen diffusion rate from 6.2 x 10^{-8} to 7.9 x 10^{-8} g cm^{-2} min^{-1} was associated with a remarkable decrease of 147 ppm in alcohol concentration. Increasing the oxygen supply to 23.7 x 10^{-8} g cm^{-2} min^{-1} almost eliminated the alcohol in the exudate samples. These data are considered evidence that the alcohol dehydrogenase reaction in the plant is extremely sensitive to aeration properties of the soil. The percentage of roots below the water table accompanying each treatment was included to permit comparison with data in Table 5.

Table 4. Effect of soil oxygen stress on ethanol concentration of xylem exudates twelve hours after treatment.

Water table position	Roots below water table	Oxygen diffusion rate	Conc. C_2H_5 OH
	%	g x 10^{8} cm^{-2} min^{-1}	ppm
Surface	100	6.2	291
-2.5 cm.	80	7.9	144
-6.25 cm	48	23.7	15
L.S.D. P.05			69

Intact Plants with Shallow Roots

Description of Experiment: Examination of the root distribution in previous experiments showed an increased

concentration along the side of the pot but vertical distribution was uniform so that flooding to the surface, 2.5 and 6.25 cm resulted in 100, 80 and 48% of the roots being submerged. It seemed possible that observed variations in ethanol concentration might be a reflection of the portion of the roots submerged rather than variation in the oxygen status of the surface soil. In order to test this possibility a tension table of the type described by Leamer and Shaw (23) was incorporated into the light chamber (Fig. 3).

Plants were grown in 3.5 cm of soil in shallow trays until three flowers opened. One end of the tray was removed, and four plants, complete with roots and soil, were slipped onto the tension table. The first series of four plants was flooded to the surface, submerging 100% of the roots as before. The second and third group of plants were subjected to water tables 2.5 and 6.25 cm below the surface resulting in 30% and none of the roots below the water table respectively. Oxygen diffusion measurements were obtained two centimeters below the soil surface as before.

Results: The ethanol concentrations reported in Table 5 were almost identical to those obtained with a quite different root distribution in experiment 4 (Table 4). This was taken as evidence that changes in ethanol concentration

reflected the oxygen status of the surface soil rather than root distribution with respect to the water table. Exposure of even a small portion (20%) of the total root volume to improved aeration resulted in a marked reduction in ethanol production.

Table 5. Effect of soil oxygen stress on ethanol concentration of xylem exudates twelve hours after treating plants grown with shallow roots.

Water table position	Roots below water table	Oxygen diffusion rate	Conc. C_2H_5 OH
	%	g x 10^8 cm^{-2} min^{-1}	ppm
Surface	100	6.2	257
-2.5 cm	30	9.3	109
-6.25 cm	0	25.9	14
L.S.D. P. 05			77

Discussion and Conclusion Regarding The Effect of Soil Aeration on Ethanol Accumulation

Soil physicists have long sought a satisfactory means of determining the critical level of soil aeration for optimum plant growth. It will be shown in experiments described later that growth was depressed by alcohol supplied in nutrient solution at concentrations similar to those

found in exudate samples. With these points in mind the alcohol concentration of the exudates was compared with the corresponding oxygen diffusion rate (Tables 4 and 5) in an attempt to define the soil oxygen status associated with the presence of ethanol in the plant. Only data from plants remaining intact until sampling time and treated for twelve hours at constant light intensity were used in this comparison. Soil oxygen diffusion rates and ethanol concentration of three plants grown without a water table were included to extend the range.

The plot of alcohol concentration versus oxygen diffusion rate (Fig. 7) indicated that the amount of alcohol produced increased sharply when the oxygen diffusion rate fell below about 20×10^{-8} g cm^{-2} min^{-1}. No alcohol was produced at O.D.R. values in excess of about 38×10^{-8} g cm^{-2} min^{-1}.

All oxygen diffusion measurements were made two centimeters below the soil surface so that they would reflect the influence of small changes in water table position. It is probable that the critical value associated with appearance of ethanol would be lower if the measurements were made at greater depth. The appearance of ethanol in plants grown in soil with physical properties different than those

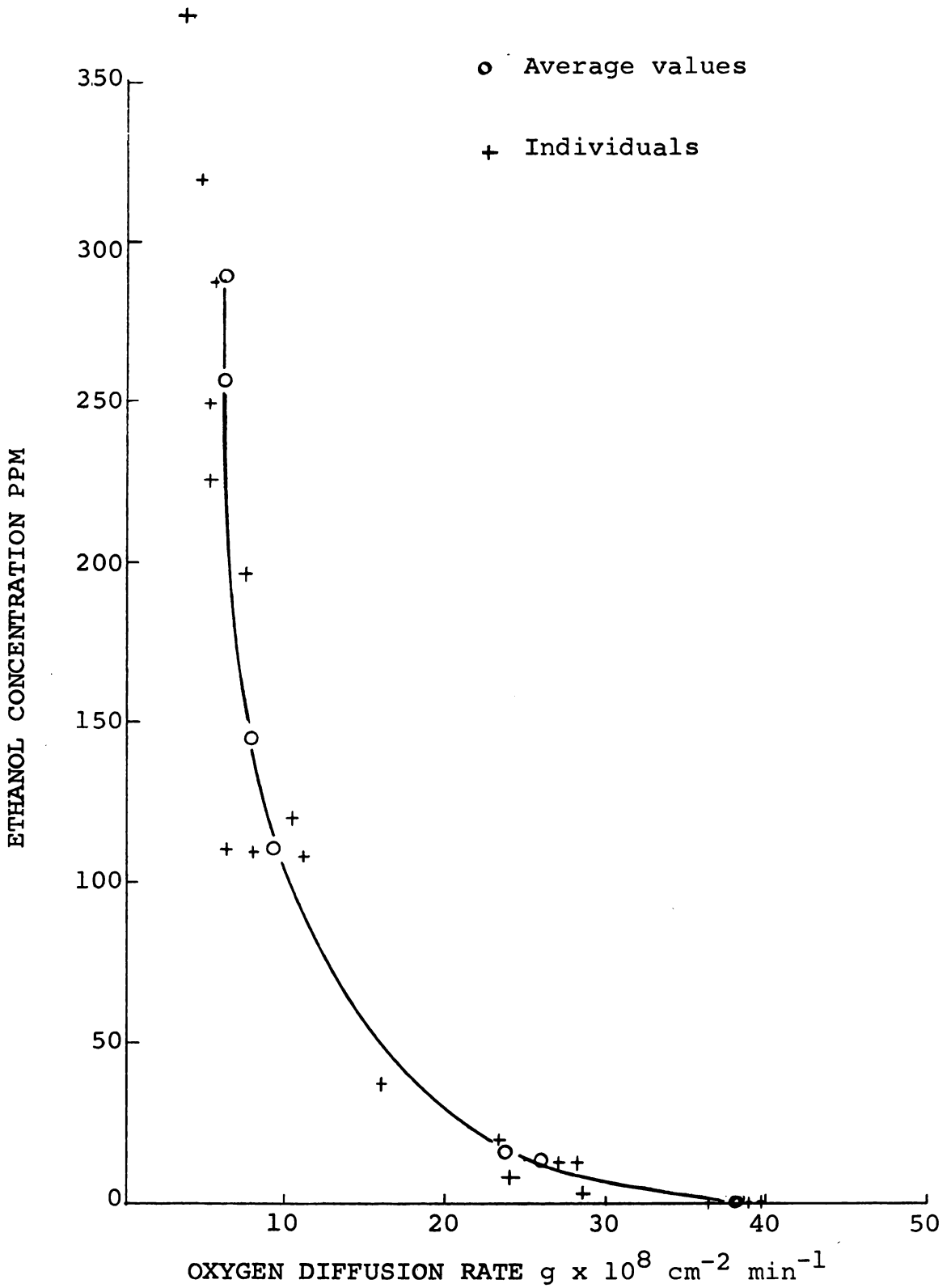


Figure 7. Relationships of soil aeration to ethanol concentration of xylem exudates.

of the soil used in this study would no doubt be associated with a different water table level. However, because the oxygen supplied to the root is a function of the rate of diffusion through the moisture film (26), the critical oxygen diffusion rate for ethanol accumulation in tomato plants should be the same for all soils.

Although this study has not clearly established that maximum growth rate was associated with zero alcohol concentration in the exudates, the data do suggest this possibility. Firm establishment of a critical O.D.R. value for optimum plant growth would greatly enhance our ability to evaluate soil-plant interrelationships. The integrated effects of the soil's physical characteristics can be measured with the platinum microelectrode. Van Doren (28) reviewed attempts to arrive at such a critical value from crop yield measurements. The values reported to date vary from 20 x 10^{-8} g cm^{-2} min^{-1} in work by Letey et al (27) to 70 x 10^{-8} g cm^{-2} min^{-1} (10).

The author is unaware of any previous attempts to utilize a relationship between soil aeration and biochemical reactions within the plant as a means of determining critical values for soil aeration. The possible number of plant reactions which could theoretically be affected by the

aeration status of the soil are numerous and some one of them may well be more sensitive to changes in soil aeration than the alcohol dehydrogenase reaction studied here. Any reaction which utilized reduced pyridine nucleotides as substrate material could be enhanced by oxygen stress. On the other hand any equilibrium reaction in which reduced pyridine nucleotides are reaction products could theoretically be depressed. Furthermore, any process which uses as a reactant a metabolite produced in a reaction involving either oxidized or reduced forms of the pyridine nucleotides could be influenced by oxygen stress. Reactions which produce metabolic end products which are not further metabolized are logical candidates for study. The fate of ethanol produced in anaerobic tissue is not fully understood. Cossins and Turner (11) recovered a portion of the ethanol produced by germinating pea seedlings in the vapor form. During dissipation of the ethanol, oxygen uptake and carbon dioxide release were increased but the carbon dioxide output accounted for only twenty-five percent of the alcohol consumed. Kenefick (22) found a portion of the alcohol dissipated from intact sugar beets in the vapor form. Lowe and James (29) supplied C^{14} labeled ethanol to carrot tissue but observed none of the label in the carbon dioxide or organic acids

despite increased respiration rates. Gudjonsdotter and Burstrom (14) working with excised wheat roots reported a growth promoting effect of low molecular weight alcohols at low concentrations but a toxic effect at concentrations from 10^{-1} to 10^{-3} molar. The highest ethanol concentration observed in this study approximated 6.5×10^{-3} molar. Although the plant material used and experimental conditions differed greatly from those employed by Gudjonsdotter, ethanol toxicity seemed possible.

The appearance of ethanol in xylem exudates is clear evidence of inadequate supplies of soil oxygen. However, it has not been established that the absence of ethanol constitutes evidence of optimum oxygen supplied to the plant tissue.

Toxicity of Ethanol in Nutrient Solutions

Description of Experiment

Plants were grown in four inch pots for a period of three weeks following emergence, at which time the roots were washed free of soil and the plants transferred to a water culture using Hoagland (19) nutrient solution. The medium was aerated vigorously during the course of the experiment. Two days after placing the plants in nutrient

solution, ethyl alcohol was added to the medium to provide the following treatments: (a) control - no ethanol, (b) 50, (c) 200 and (d) 400 parts per million of ethanol. Each treatment appeared in triplicate. Fresh weight of the whole plant was measured at the time the treatments were established and again five days later at the conclusion of the experiment. Each of the nutrient media was replaced with freshly prepared solutions every forty-eight hours. Using another group of plants treated in the same manner, the alcohol concentration of the xylem exudates was measured twelve hours after initiation of the experiment.

Results and Discussion

Ethanol supplied to young tomato plants in nutrient solution at concentrations of 0, 50, 200 and 400 ppm produced xylem exudate concentrations of 0, 45, 170 and 360 ppm twelve hours later. Table 6 shows that the increase in fresh weight was reduced by ethanol concentrations of 200 and 400 ppm. Although the reduction at 50 ppm is not statistically significant at a probability of .05 a trend in this direction exists. These data are considered evidence that the physiological concentration of ethanol observed in exudates of plants grown in flooded soil (Tables 4 and 5) was sufficient to be toxic.

Table 6. Effect of ethanol in nutrient solution on the concentration of ethanol in xylem exudates and growth of young tomato plants.

$C_2 H_5 OH$ concentration		
Media	Xylem exudate*	Weight increase**
PPM	PPM	% of initial
0	0	151
50	45	123
200	170	104
400	360	87
L.S.D. P .05		46

*Data from one replication only.

**Ave. three replications.

It seems quite possible that the permanent damage suffered by plants subjected to short term but severe oxygen stress (Table 2), imposed by flooding the soil, may be due to the toxic effects of the ethanol produced by fermentation. However, it is unlikely that the reduced growth resulting from a water table at ten centimeters below the surface could be due to ethanol toxicity because ethanol accumulation was almost eliminated when the water table was lowered to 6.25 cm. This latter injury resulting from continued exposure to only slight stress probably involves restricted oxidative phosphorylation and the many reactions which could

be influenced by reduced supplies of high energy phosphate bonds.

The Effect of Oxygen Saturated Flood Water on Ethanol Accumulation

Description of Experiment

If the alcohol measured in the xylem exudates developed in response to an oxygen stress rather than excess water *per se* the concentration observed should be reduced by bubbling molecular oxygen through the flood water. This theory was tested by comparing the alcohol concentration of plants flooded for twelve hours with (a) water equilibrated with the atmosphere and (b) water through which oxygen was bubbled continuously during the experimental period. Six plants were subjected to each of the treatments. The plants with roots and soil intact were removed from the clay pots before flooding to ensure that the pot would not restrict oxygen diffusion.

Results and Discussion

The average ethanol concentration of xylem exudates from six plants flooded for twelve hours with water through which oxygen was bubbled was 45 ppm. The corresponding value for plants flooded with water in equilibrium with

the atmosphere was 173 ppm. Reduced ethanol concentration in the presence of molecular oxygen indicated, as expected, that the flooding treatments enhanced ethanol production by restricting oxygen supply. Failure to completely eliminate ethanol production with oxygen was probably due to restricted movement of the oxygen from the flood water into the block of soil.

The Influence of Light Intensity on Ethanol Accumulation

Description of Experiments

Two observations prompted the following study. The first concerns the observation that field grown plants flooded by excessive rainfall were debilitated more severely on bright sunny than on cloudy days. The second observation concerns an apparent variation in measured ethanol concentration on bright versus overcast days. Two light treatments were established by utilizing the light chamber and the semi-darkness obtained by placing the potted plants in paper cartons and covering with a dark cloth. These were compared with "decapitated" plants placed on the laboratory bench. Immediately after subjecting the plants to their respective light conditions water was added until the water table reached the surface of the soil. After twelve

hours had elapsed, samples were collected and analyzed for ethyl alcohol. Each treatment was replicated four times.

The data obtained in this experiment prompted a second one. Due to the mutually interdependent nature of the two experiments they are described and discussed jointly. In the second experiment the following four treatments were established in quadruplicate (a) plants flooded in the light, (b) plants flooded in the light and girdled to sever the phloem, (c) plants flooded in the dark, and (d) plants flooded in the dark and girdled to sever the phloem.

Results and Discussion

Table 7 shows that ethanol accumulation was greater in "decapitated" plants than intact plants flooded in the light. A further reduction was observed for intact plants flooded in the dark. Soldatenkov (40) reported that the leaves of corn and bean plants protected the roots from the harmful effects of anaerobiosis. He concluded that the protective action probably consisted of providing atmospheric oxygen through the conducting system. If such a mechanism operated in this study the ethanol concentration should be lower in plants flooded in the light than those flooded in the dark. The oxygen released through photosynthesis should

Table 7. The effect of light intensity on the ethanol concentration in xylem exudates of flooded tomato plants.

Treatment	C_2H_5OH ppm
Decapitated plants flooded	445
Intact plants flooded in light	255
Intact plants flooded in dark	136
L.S.D. P .05	106

have reduced the oxygen stress. It seems likely that an alcohol disposal mechanism similar to that described by Cossins (11) and Kenefick (22) was responsible for some of the protective action of the foliage. Increased ethanol production by plants in the light may have resulted from transport of some material produced in photosynthesis to the root where it served as a substrate material for alcohol dehydrogenase. If by some unknown mechanism some of the reducing power produced by photosynthesis were transferred to the root the alcohol dehydrogenase reaction would be enhanced.

Table 8 provided some evidence for such a proposal. Girdled plants flooded in the light accumulated less ethanol than plants with an intact phloem. Plants flooded in the dark accumulated similar quantities of ethanol in both the

girdled and intact plants.

Table 8. The influence of girdling on the ethanol concentration of xylem exudates from flooded plants.

Treatment	C_2H_5 OH ppm
Flooded in light	195
Flooded in light and girdled	104
Flooded in dark	138
Flooded in dark and girdled	115
L.S.D. P .05	70

The increased alcohol observed in the light may explain the observation that, when field grown plants are flooded, the damage is more severe if followed by sunny rather than overcast days. This is probable because the ethanol concentrations observed in flooded plants were sufficient to be toxic.

Effects of Soil Flooding on the Distribution of C^{14} in Metabolic Intermediates

Description of Experiments

Plants which had fixed $C^{14}O_2$ for a period of two hours were harvested so as to provide experiments of 18, 30, 54 and 78 hours duration, measured from the initiation of $C^{14}O_2$ to harvest. One half of the plants in the 18 and

30 hour experiments were flooded to the surface of the soil for 12 hours. One half of those in the 54 and 78 hour experiments were flooded for 36 and 60 hours respectively. The remaining plants were watered normally and served as aerobic controls.

Data from plants killed eighteen hours after supplying labeled carbon dioxide were obtained in a single experiment. All experiments of longer duration were conducted in duplicate but statistical analyses of the variability evident in Appendix Tables C, D and E werenot justified. No attempt was made to account for C^{14} incorporated into nonextractable tissue, evolved in respiration or contained as ethanol in flooded plants. Assuming equal fixation of carbon dioxide by all plants, those in the eighteen-hour experiment received 0.131 millicuries whereas plants in the thirty hour experiment received 0.121 millicuries in one replication and 0.171 in the other. All plants in the fifty-four and seventy-eight hour experiments received C^{14} at the rate of 0.118 millicuries per plant.

Results and Discussion

The total counts found in the combined alcohol-water extract, as measured with the end window counter, are

reported in Table 9. Differences in amount of label supplied complicated interpretation of changes in extractable C^{14} with increasing duration of the experiments. In spite of this anomaly there can be little doubt that the proportion extractable decreased with time. Within experiments of any given duration the flooded and "normal" plants were subjected to the same atmospheric concentration of $C^{14}O_2$. In all but one of the twelve comparisons shown in Table 9 the amount of extractable C^{14} was greater in flooded than normal plants. The magnitude of this increase did not seem to change with

Table 9. Total C^{14} in alcohol-water extracts of tomato plants.

Experiment hours	Flooded hours	Counts per second			
		Roots	Stems	Leaves	Total
18	0	2,368	2,255	24,018	28,641
	12	4,494	4,860	30,414	39,768
30	0	2,295	5,935	22,700	30,930
	12	2,371	4,320	24,637	31,328
54	0	454	1,604	10,550	12,608
	36	892	2,259	15,105	18,356
78	0	641	1,257	9,550	11,248
	60	789	2,325	13,590	14,704

the duration of the flooded treatments. Although C^{14} present in the non-extractable form was not measured, it is probable

that the reduced amount extracted from "normal" plants was a consequence of greater incorporation into structural tissue of "normal" than flooded plants.

The distribution of C^{14} among compounds detected on the chromatograms is shown in Appendix Tables C, D and E. Each value represents the C^{14} found in the corresponding compound expressed as percent of the total found on the chromatogram. Averaged data for those compounds which appeared to be affected most by the flooding treatments are summarized in Tables 10, 11 and 12. Known biological reaction sequences account for the presence of each of the compounds listed. Photosynthetic reactions (2, 18) along with those of fermentation and the tricarboxylic acid cycle account for the presence of glucose, fructose, sucrose, malate, citrate, succinate and α ketoglutarate. Malate may also be formed by carboxylation of pyruvate or phosphoenolpyruvate (13). Glutamate is formed in the reaction between ammonia and α ketoglutarate catalyzed by L-glutamic dehydrogenase. Aspartate and alanine could arise from transamination reactions between glutamate and the appropriate keto acids. The lactic dehydrogenase reaction has been shown previously. Schales et al (37) isolated L-glutamic decarboxylase from higher plants and showed that

pyridoxal phosphate was the prosthetic group. Subsequently numerous workers (4, 9, 20, 43, 49) have studied the distribution and activity of the decarboxylase in a variety of plant tissues. The products of the reaction are gamma amino butyric acid and carbon dixoide. Fruton and Simmonds (13) reviewed literature dealing with the formation of glutamine from glutamic acid, ammonia and ATP. Recently Lynen (30) reported the synthesis of saturated fatty acids from acetyl coenzyme A, reduced pyridine nucleotides and ATP. Comparison of the C^{14} distribution in extracts of flooded with that of normal plants showed that a decrease in the proportion found in carbohydrates was associated with an increase in the amount found in the lipid fraction.

Table 10. Percent distribution of C^{14} in alcohol-water extracts of tomato roots.

Experiment (hrs)	18		30		54		78	
Flooded (hrs)	0	12	0	12	0	36	0	60
	%	%	%	%	%	%	%	%
Glucose	5.5	15.2	10.6	7.5	0.0	3.2	0.0	4.6
Fructose	16.3	20.5	19.2	14.4	0.0	3.2	0.0	5.5
Sucrose	4.7	18.5	10.5	8.4	0.0	3.2	0.0	4.5
Malate	15.8	8.4	16.3	10.9	11.4	7.8	5.5	8.8
Citrate	3.9	2.5	7.8	8.7	2.8	7.4	3.3	8.9
Succinate	3.2	1.8	0.0	1.8	0.0	1.7	0	0
Glutamate	3.7	1.7	4.6	2.5	2.8	2.7	2.1	2.4
α ketoglutarate	2.2	1.4	1.5	2.9	0.8	1.5	2.2	1.8
Glutamine	4.3	2.3	4.6	1.2	3.0	0.6	0.0	0.0
Aspartate	1.5	0.5	1.0	0.0	0.0	1.3	0.0	0.0
Lactate	0.0	0.7	0.0	0.2	0.0	0.0	0.0	0.0
Alanine	0.0	4.3	0.0	0.6	0.0	0.0	0.0	0.0
γ amino butyrate	0.0	3.9	0.0	4.2	0.0	3.0	0.0	4.5
Lipids	18.7	10.9	11.8	22.6	53.2	35.4	39.4	34.7

Table 11. Percent distribution of C^{14} in alcohol water extracts of tomato stems.

Experiment (hrs)	18		30		54		78	
Flooded (hrs)	0	12	0	12	0	36	0	60
	%	%	%	%	%	%	%	%
Glucose	2.2	21.9	27.2	13.9	0.0	3.5	0.0	26.7
Fructose	3.2	13.9	12.8	9.6	0.0	1.6	0.0	1.8
Sucrose	3.4	5.1	5.4	4.7	1.5	2.0	0.0	13.9
Malate	45.0	21.0	16.9	25.4	7.6	21.4	0.0	13.7
Citrate	12.2	6.0	11.5	7.0	17.8	11.6	33.9	21.4
Succinate	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Glutamate	2.2	2.1	2.6	2.7	3.4	1.7	3.4	0.0
α ketoglutarate	1.2	1.4	0.4	1.9	0.0	0.5	0.0	0.8
Glutamine	2.2	1.4	3.9	4.2	8.9	10.0	10.4	6.3
γ amino butyrate	0.0	1.4	0.5	2.0	1.5	2.0	0.8	1.5
Lipids	8.8	7.3	8.1	14.7	31.7	19.7	21.8	0.0

Table 12. Percent distribution of C^{14} in alcohol water extracts of tomato leaves.

Experiment (hrs)	18		30		54		78	
Flooded (hrs)	0	12	0	12	0	36	0	60
	%	%	%	%	%	%	%	%
Glucose	2.8	4.9	10.6	5.2	0.5	1.4	1.8	1.8
Fructose	5.3	7.6	17.8	9.7	1.6	2.4	3.7	4.3
Sucrose	1.4	2.5	4.6	2.8	1.2	1.9	1.8	2.1
Malate	45.0	43.8	27.3	24.0	18.7	20.5	15.7	21.5
Citrate	16.2	12.8	10.6	20.0	25.9	35.7	32.8	40.6
Glutamate	3.8	5.0	3.8	3.4	2.4	3.4	3.3	2.7
α ketoglutarate	tr	0.0	0.8	0.7	0.4	0.0	0.0	0.0
Glutamine	0.0	0.7	1.5	0.9	5.4	1.1	3.9	0.5
Lipid	5.6	4.9	7.3	10.5	29.2	16.3	11.5	9.7

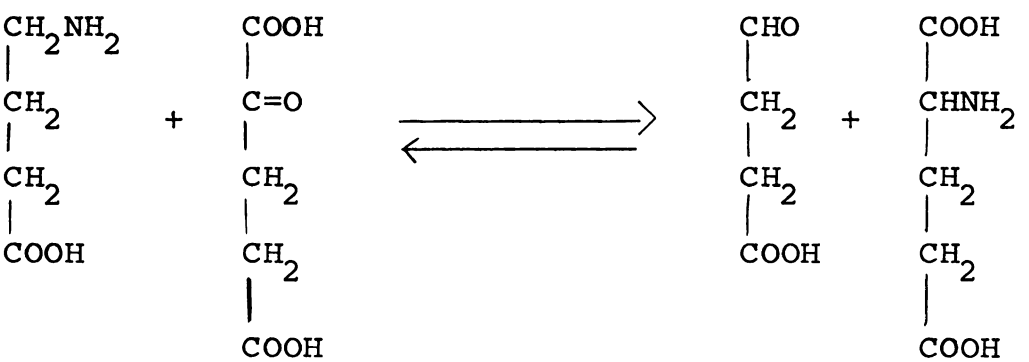
The flooding treatment increased the amount of C^{14} found in carbohydrates of the eighteen, fifty-four and seventy-eight hour experiments but the reverse was observed in the thirty hour experiment. The actual mechanism by which the flooding treatment influenced the metabolism of carbohydrate C^{14} are not defined in these experiments. However, interpretation of these data in the light of facts reported in the literature suggests some interesting possibilities. Restricted oxygen supply is known to restrict the tricarboxylic acid cycle reactions and cause a build up of pyruvic acid (5). Subsequent metabolism of pyruvate would then serve as a means of controlling the rate of carbohydrate metabolism. Thus ethanol production under conditions of oxygen stress would enhance mobilization of carbohydrate C^{14}. It seems possible that lipid synthesis, utilizing reduced pyridine nucleotides and two carbon units from decarboxylation of pyruvate served as a second means of promoting pyruvate utilization under conditions of soil oxygen stress. Fatty acid synthesis is ATP dependent and the extent of this reaction may have been determined by the supply of ATP available. In the eighteen-hour experiments, the flooding treatment was initiated in the evening and continued through the night. Both the flooding treatment and the presence of

darkness would reduce tht total ATP synthesis. In addition, it was shown in Tables 7 and 8 that less ethanol was produced by plants flooded in the dark than when flooded in the light. Thus in a qualitative way, reduced synthesis of ethanol and lipid materials could account for the fact that carbohydrate C^{14} was higher than "normal" in flooded plants of the eighteen-hour experiment but lower than "normal" in the thirty-hour experiments. Fifty-four and seventy-eight hours after supplying the $C^{14}O_2$, the roots and stems of aerobic plants were completely devoid of carbohydrate C^{14} while small amounts of C^{14} remained in the flooded plants. In these experiments as in others the removal of C^{14} from the carbohydrate fractions was accompanied by an increase in the amount found in the lipids. However, the theories proposed would not explain these facts unless the toxic effect of ethanol produced in plants flooded in excess of twelve hours was sufficient to offset photophosphorylation and the Pasteur effect (46).

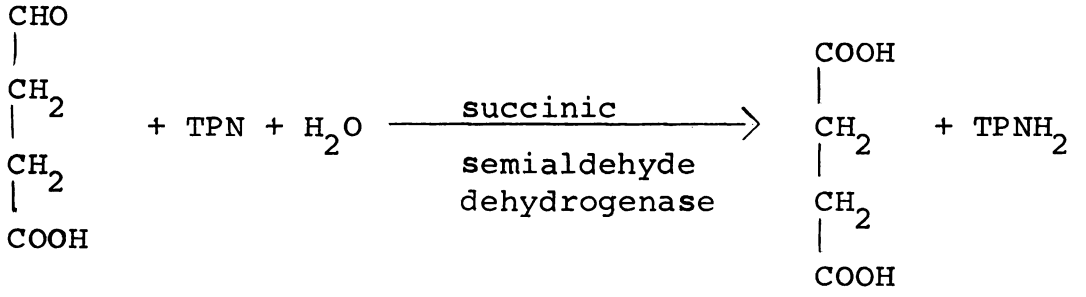
Labeled alanine appeared in the root extract of flooded plants eighteen and thirty hours after supplying $C^{14}O_2$. This amino acid was probably formed by transamination between glutamic and pyruvic acids. Failure to detect labeled alanine in experiments longer than thirty

hours indicated that it was produced only during the early stages of flooding and was rapidly metabolized. More of the label appeared in glutamine of the normal aerobic plants than in the flooded plants. This may be due to the fact that glutamine synthesis is ATP dependent (13).

Root extracts of all flooded plants contained labeled gamma amino butyric acid whereas none of the label appeared in this compound in root extracts of "normal plants." Naylor and Tolbert (34) observed an increase in the label found in gamma amino butyric acid of barley leaves under anaerobic conditions. Reference has already been made to the numerous literature reports of γ amino butyrate formation in plants by decarboxylation of glutamic acid. The enzyme has been isolated and purified but there is no evidence that its activity is oxygen dependent. Working with brain tissue Bessman et al (7) demonstrated a transamination reaction between γ amino butyrate and α keto glutarate.

1) 

Subsequently several workers (1, 21, 38) have confirmed the transamination reaction and succeeded in isolating a succinic semialdehyde dehydrogenase which catalyzed the oxidation of succinic semialdehyde to succinic acid.

2) 

In the <u>in vitro</u> system where the oxidized form of TPN was supplied, the enzyme was equally active in air or helium (21). However, the appearance of γ amino butyrate in the root extracts of flooded plants in the present study may reflect the lack of oxidized forms of the pyridine nucleotides under oxygen stress, in keeping with the proposal by McKhann and Tower (31) that competition for DPN may determine which pathway of α ketoglutarate oxidation is favored. In addition, the presence of ethanol in the flooded plants may promote accumulation of γ amino butyrate as observed by Häkkinen and Kulonen (16, 17) in brain tissue.

Tables F and G of the Appendix rates the intensity of color produced when the chromatograms were sprayed with ninhydrin. Aspartate and glutamate were rated higher in

roots of aerobic than flooded plants. No clear trend was observed in stem extracts. The intensity of the glutamine spots was approximately the same for both aerobic and flooded plants. In most cases the γ amino butyrate and alanine spots were more intense in flooded than in non flooded plants. The remaining amino acids listed appeared relatively infrequently and do not reflect a treatment effect.

The appearance of unlabeled γ amino butyrate in the root extracts of both aerobic and flooded plants, while the labeled compound appeared only in the flooded plants, suggests that the substrate was not a labeled compound for the aerobic plant but was labeled in the flooded plant. It seems probable that the glutamic acid precursor came from hydrolysis of proteins in aerobic plants and out of the tricarboxylic acid cycle in flooded plants. The same line of reasoning applies to alanine.

Gamma Amino Butyric Acid Toxicity

Description of Experiment

The observed tendency for γ amino butyric acid to accumulate in the roots and stems of flooded plants prompted additional studies with this compound. To test possible toxic effects of this compound, the roots of intact tomato

plants were washed free of soil and placed in complete Hoagland (19) nutrient solution. Forty-eight hours later the nutrient media were replaced by freshly prepared stock to which γ amino butyric acid was added to give the following concentrations 0, 10^{-3}, 10^{-4}, and 10^{-5} M. These media were replaced with freshly prepared solutions every forty-eight hours. The fresh weight of the plants was recorded at the start of the experimental period and again five days later, at the end of the experiment. The experiment was conducted with five replications of each treatment. Identical experiments were conducted using nicotiana tabacum L. 'Burley No. 1' except that only three replications were used (Tables 13 and 14).

Table 13. Growth of tomato plants in Hoagland solution with γ amino butyric acid.

Concentration γ amino butyrate	Replication I	II	III	IV	V	Mean
	Fresh wt. increase as % of initial					
0	368	300	99	116	202	217
10^{-5} M	350	310	102	121	148	206
10^{-4} M	387	276	97	119	178	211
10^{-3} M	370	256	49	122	105	180
L.S.D. P .05						N.S.

Table 14. Growth of young tobacco plants in Hoagland solutions with γ amino butyric acid.

Concentration γ amino butyrate	Replication I	II	III	Mean
	Fresh wt. increase as % of initial			
0	154	151	191	165
10^{-5} M	103	155	221	159
10^{-4} M	140	158	184	161
10^{-3} M	135	150	190	158
L.S.D. P .05				N.S.

Results and Discussion

Data in Tables 13 and 14 showed that the presence of γ amino butyric acid in the nutrient solution had no significant toxic effect on the growth of tomato or tobacco plants within the range of concentrations used.

Gamma Amino Butyric Acid as a Source of Nitrogen

Description of Experiment

In order to test the possibility of using γ amino butyric acid as the sole nitrogen source for tomato plants the following solutions were prepared: (a) normal Hoagland solution, (b) Hoagland solution at one half the normal concentration and (c) Hoagland no-nitrogen solution plus γ amino butyric acid to provide the same molarity with respect to nitrogen as in (a). Fresh solutions were prepared every

forty-eight hours during the five day experimental period. The difference between initial and final fresh weight was used as a measure of treatment response.

Results and Discussion

The five-day weight increase of tomato plants was 84, 94, and 25 percent of initial plant weight for normal strength, one half normal strength Hoagland solution and for a nutrient solution using γ amino butyric acid as the sole source of nitrogen. Thus γ amino butyric acid supplied in nutrient solution was not satisfactory as the sole source of nitrogen. Absorption into the plant roots or failure to transaminate at a sufficient rate may have been responsible; however, no attempt was made to evaluate these possibilities. The nutrient solution was tested with diphenylamine at the beginning and end of the experiment in order to determine whether nitrification had taken place in the nutrient media. All tests proved negative indicating no nitrification of the γ amino butyric acid in solution.

SUMMARY

Soil oxygen stress imposed by flooding the soil for periods of twenty-four hours was shown to reduce ultimate yield of tomato foliage and fruit. The effect was most severe when the plants were flooded three or four weeks post emergence. Yield of foliage was also reduced when the water table was maintained 2.50 and 10 cm below the surface for a period of two weeks. The magnitude of the yield reduction increased with the severity of the oxygen stress.

Ethanol accumulation in xylem exudates of tomatoes was dramatically influenced by variations in the oxygen status of the soil. Ethanol appeared when the oxygen diffusion rate, measured two centimeters below the surface, fell below 38×10^{-8} g cm^{-2} min^{-1}. The concentration of ethanol increased sharply as the oxygen status of the soil was further reduced. Ethanol supplied in nutrient solution at concentrations similar to those found in xylem exudates of flooded plants was shown to be toxic. It was suggested that the permanent injury caused by severe short term flooding may have resulted from ethanol toxicity.

The ethanol concentration in xylem exudates of flooded

plants was greater when the plants were subjected to high light intensity than in reduced light. It was suggested that this factor might explain the increased debilitation of flooded plants observed on bright sunny days.

Less C^{14} was extracted from aerobic control plants than the flooded plants. This was assumed to be a consequence of greater incorporation into non-extractable forms by the aerobic plants. Oxygen stress imposed by flooding the root zone was reflected in numerous changes in distribution of the C^{14} in roots, stems and leaves of tomato plants. One of the outstanding changes observed was the appearance of C^{14} labeled gamma amino butyric acid in the roots of flooded plants while none was found in roots of aerobic plants. However, non labeled γ amino butyrate was found in the extracts of aerobic roots although in lesser amounts than in roots of flooded plants. γ amino butyrate was shown to be non toxic when fed in nutrient solutions but was not satisfactory as the sole source of nitrogen.

Comparison of C^{14} distribution in flooded and aerobic plants showed that a decrease in carbohydrate C^{14} was always accompanied by an increase in lipid C^{14}. It was suggested that, under some conditions at least, incorporation of carbon into lipid materials served to

remove some of the pyruvate accumulated under oxygen stress.

The influence of many of the chemical changes associated with oxygen stress on the overall growth of the plant could not be evaluated in these experiments.

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APPENDIX

Table A. Ethanol concentration of xylem exudates of decapitated tomato plants subjected to soil oxygen stress at the bloom stage.

Water table position	Log ethanol concentration ppm				Mean
	I	II	III	IV	
	4 hours after treatment				
Surface	1.9138	2.4440	2.1761	2.1139	2.1619
-2.50 cm	1.4150	2.1761	2.2304	1.3424	1.7909
-6.25 cm	0.9031	1.2553	1.0000	0.6021	0.9401
	L.S.D. P .05				0.3580
	8 hours after treatment				
Surface	2.1553	2.4742	2.5740	2.5441	2.4369
-2.50 cm	2.0128	2.2672	2.4232	1.7782	2.1203
-6.25 cm	1.2304	1.3802	0.8451	0.9542	1.1025
	L.S.D. P .05				0.4282
	12 hours after treatment				
Surface	2.5502	2.5911	2.6464	2.5798	2.5918
-2.50 cm	2.0969	2.2856	2.5224	1.8129	2.1794
-6.25 cm	1.3434	1.3979	1.2041	0.4771	1.1056
	L.S.D. P .05				0.4135
	24 hours after treatment				
Surface	2.5798	2.6990	2.6464	2.5635	2.6222
-2.50 cm	2.3617	2.2945	2.5224	1.8129	2.2479
-6.25 cm	1.5441	1.6628	1.1761	0.6990	1.2705
	L.S.D. P .05				0.0211

Table B. Ethanol concentration of xylem exudates of decapitated tomato plants subjected to soil oxygen stress prior to blooming.

Water table position	log ethanol concentration ppm I	II	III	Mean
	4 hours after treatment			
Surface	0.9031	1.2788	1.2788	1.1535
-2.50 cm	0.9031	1.1139	0.6021	0.6064
-6.25 cm	0.0	0.0	0.6021	0.2007
	L.S.D. P .05			0.7551
	8 hours after treatment			
Surface	1.7559	1.3010	1.8751	1.3107
-2.50 cm	1.1461	1.1139	1.4472	1.2357
-6.25 cm	0.0	0.0	0.6990	0.2330
	L.S.D. P .05			0.8189
	12 hours after treatment			
Surface	2.1761	1.4314	2.3222	1.9766
-2.50 cm	1.0792	1.6812	1.2304	1.3302
-6.25 cm	0.0	0.3010	0.4771	0.2593
	L.S.D. P .05			0.9161
	24 hours after treatment			
Surface	1.8865	1.4914	2.4698	1.9492
-2.50 cm	1.4150	1.4314	1.3979	1.4147
-6.25 cm	0.0	0.0	0.6021	0.2007
	L.S.D. P .05			0.6273

Table C. Percent distribution of C^{14} in alcohol-water extracts of tomato roots from plants grown with normal soil moisture and in soil flooded for varying periods of time.

Duration of expt.(hrs)	18		30		54		78							
Hours flooded	0	12	0	12	0	12	0	36	0	36	0	60	0	60
	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Glucose	5.5	15.2	6.4	6.2	14.8	8.8	0	3.1	0	3.2	0	3.6	0	5.5
Fructose	16.3	20.5	17.7	14.7	20.6	14.0	0	3.1	0	3.2	0	6.4	0	4.5
Sucrose	4.7	18.5	6.9	5.8	15.0	10.8	0	5.4	0	3.7	0	3.4	0	5.5
Malate	15.8	8.4	17.1	12.9	15.5	8.8	10.7	7.6	12.1	7.9	11.0	9.2	0	8.3
Citrate	3.9	2.5	5.0	4.9	10.5	12.5	5.7	9.4	tr.	5.4	0	10.0	6.7	7.8
Glutamate	3.7	1.7	2.4	1.5	6.7	3.5	1.7	2.9	3.8	2.5	0	2.6	4.1	2.2
α ketoglutarate	2.2	1.4	2.9	3.4	tr.	2.3	0	1.8	1.6	1.1	0	2.2	4.4	1.3
Glutamine	4.3	2.3	7.8	2.3	1.4	0	3.5	0	2.5	1.2	0	0	0	0
Aspartate	1.5	0.5	1.9	0	0	0	0	1.4	0	1.1	0	0	0	0
Succinate	3.2	1.8	0	1.7	0	1.9	0	3.3	0	0	0	0	0	0
Lactate	0	0.7	0	0.4	0	0	0	0	0	0	0	0	0	0
Glycolate	3.5	0.5	0	1.4	0	0	0	0	0	0	0	0	0	0
Glycerate	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Alanine	0	4.3	0	1.1	0	0	0	0	0	0	0	0	0	0
γ amino butyrate	0	3.9	0	4.0	0	4.4	0	2.9	0	3.0	0	4.4	0	4.5
P-esters	tr.	1.2	0	0	0	0	8.8	7.2	tr.	6.5	11.7	6.8	12.3	5.2
Lipid	18.7	10.9	23.5	26.8	0	18.4	50.3	29.8	56.0	40.9	42.8	34.4	36.0	35.0
Miscellaneous	8.3	2.9	1.9	6.3	3.0	4.6	1.7	2.7	4.5	5.1	8.5	3.4	7.0	8.8
Origin	8.4	2.8	6.7	5.6	13.0	10.4	17.6	19.4	20.2	15.5	25.9	14.4	29.6	12.8

Table D. Percent distribution of C^{14} in alcohol-water extracts of tomato stems from plants grown with normal soil moisture and in soil flooded for varying periods of time.

Duration of expt. (hrs)	18		30		54		78							
Hours flooded	0	12	0	12	0	12	0	36	0	36	0	60	0	60
	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Glucose	2.2	21.9	33.4	11.1	21.0	16.7	0	3.4	0	4.3	0	14.6	0	38.8
Fructose	3.2	13.9	14.5	8.0	11.0	11.2	0	1.2	0	2.0	0	3.6	0	0
Sucrose	3.4	5.1	3.8	3.8	7.0	5.6	3.0	2.3	0	1.6	0	3.8	0	0
Malate	45.0	21.0	32.0	48.5	1.8	2.3	5.9	17.2	9.2	25.6	0	20.6	0	6.8
Citrate	12.2	6.0	0	2.0	21.0	12.0	22.1	5.4	13.5	17.8	38.0	16.0	22.8	26.8
Glutamate	2.2	2.1	2.1	2.3	3.0	3.0	3.3	1.1	3.5	2.2	3.7	0	2.4	0
α ketoglutarate	1.2	1.4	0	3.0	0.8	0.8	0	0.9	0	0	0	1.6	0	0
Glutamine	2.2	1.4	1.6	3.2	6.1	5.1	10.7	7.0	7.2	13.0	11.0	12.5	7.7	0
Aspartate	2.6	1.3	0	0	0	0	0	1.2	0	0	0	0	0	0
Succinate	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactate	0	2.6	0	0	0	0	0	0	0	0	0	0	0	0
Glycolate	0	0.6	0	0	0	0.8	1.2	1.9	2.0	0	0	0	0	0
Glycerate	0	0	0	0.5	0	0	2.6	1.4	0	0	0	2.3	4.5	0
Alanine	0	0	0	0	0	0	0	0	0	0	0	0	0	0
γ amino butyrate	0	1.4	0	2.5	1.0	1.4	3.0	2.2	0	1.7	0	2.9	1.6	0
α-esters	2.0	1.3	0	0	1.8	0	10.2	3.1	1.8	2.7	6.6	4.0	12.0	17.5
Lipids	8.8	7.3	7.8	7.2	8.3	22.2	20.4	30.2	43.0	9.2	17.8	0	22.6	0
Miscellaneous	6.8	8.0	4.1	3.0	10.0	11.3	8.1	10.4	6.8	12.3	7.9	12.2	17.0	0
Origin	7.5	5.0	3.6	5.2	7.3	7.7	9.3	11.2	13.7	6.4	15.0	6.3	9.7	10.5

Table E. Percent distribution of C^{14} in alcohol-water extracts of tomato leaves from plants grown with normal soil moisture and in soil flooded for varying periods of time.

Duration of expt. (hrs)	18		30		54		78							
Hours flooded	0	12	0	12	0	12	0	36	0	36	0	60	0	60
	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Glucose	2.8	4.9	8.9	4.3	12.2	6.0	0	1.6	1.0	1.1	1.8	1.8	1.7	1.8
Fructose	5.3	7.6	13.2	8.3	22.3	11.1	1.3	2.4	1.9	2.3	4.1	2.0	3.3	6.6
Sucrose	1.4	2.5	2.0	1.6	7.2	4.5	1.2	1.8	1.1	1.9	1.9	1.4	1.6	2.7
Malate	45.0	43.8	29.0	31.0	25.5	17.0	22.5	9.5	14.8	31.4	20.3	18.7	11.0	24.2
Citrate	16.2	12.8	4.6	19.0	16.5	31.0	26.6	38.8	25.1	32.6	34.4	47.2	31.2	34.0
Glutamate	3.8	5.0	3.4	3.4	4.3	3.4	2.7	3.9	2.0	2.8	2.0	1.6	4.6	3.7
α ketoglutarate	tr	0	0	0	1.5	1.3	0	0	0.7	0.9	0	0	0	0
Glutamine	0	0.7	0.7	0.4	2.2	1.3	6.4	1.1	4.3	1.0	5.5	1.0	2.5	0
Aspartate	2.1	2.0	1.6	2.0	0	1.7	1.5	2.4	1.3	1.7	0	1.2	1.4	1.6
Succinate	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lactate	0	0	0.3	0.6	0	0	0	0	0	0	0	1.6	0	0
Glycolate	0.6	1.4	0.8	0.8	0	1.0	0	1.5	0	0.5	0	0	0	0
Glycerate	2.9	0	2.5	2.5	0	0	1.5	0	0	0	0	0	0	0
Alanine	0	0	0	0	0	0	0	0	0	0	0	0	0	0
γ amino butyrate	0	0	0	0	0	0	0	0	0	0	2.6	0	0	0
P-esters	1.5	0	0	0.7	0	0	3.7	4.6	5.7	4.7	7.5	5.6	10.7	3.6
Lipids	5.6	4.9	14.5	9.8	0	11.1	25.5	19.6	32.8	13.0	13.3	13.0	9.7	6.4
Miscellaneous	11.4	13.3	17.4	14.3	7.3	8.5	5.8	10.8	5.7	4.0	4.0	4.0	20.2	14.3
Origin	1.4	1.1	1.2	1.1	1.2	2.0	2.3	2.7	3.8	2.6	2.7	2.5	2.2	2.1

Table F. Rated intensity of ninhydrin positive spots on chromatograms prepared from alcohol-water extracts from roots of plants subjected to normal soil moisture and flooded for various periods of time.

Duration of expt. (hrs)	18		30		54				78					
Hours flooded	0	12	0	12*	0	12	0	36	0	36	0	60	0	60
Aspartate	2	tr	1	--	0	0	1	0	2	tr	2	1	2	1
Glutamate	2	2	2	--	2	**2**	3	2	4	3	3	2	3	3
Glutamine	2	1	1	--	1	1	2	2	2	2	2	2	2	2
γ amino butyrate	tr	2	tr	--	0	2	1	2	2	2	1	3	1	3
Alanine	1	2	tr	--	1	2	1	2	1	2	1	3	1	2
Serine	1	1	1	--	1	1	1	1	1	2	1	1	1	1
Glycine	0	tr	0	--	0	0	0	0	0	0	0	0	0	0
Cysteine	0	0	0	--	0	0	0	0	0	0	0	0	0	0
Methionine	0	0	0	--	0	0	0	0	0	0	0	0	0	0
Phenylalanine & leucine	0	0	0	--	0	0	0	0	0	0	0	0	0	tr
Threonine	0	0	tr	--	0	0	0	0	tr	0	0	0	tr	0

*The chromatogram used for co-chromatography.

Table G. Rated intensity of ninhydrin positive spots on chromatograms prepared from alcohol-water extracts from stems of plants subjected to normal soil moisture and flooded for various periods of time.

Duration of expt. (hrs)	18		30		54				78					
Hours flooded	0	12	0	12	0	12	0	36	0	36	0	60	0	60
Aspartate	2	2	2	3	0	0	0	2	2	0	1	2	2	--
Glutamate	2	2	3	3	4	3	4	3	3	4	4	3	3	--
Glutamine	1	1	3	3	4	3	4	4	3	4	4	4	4	--
γ amino butyrate	0	1	1	3	3	3	3	2	1	2	1	3	2	--
Alanine	0	1	1	2	3	2	3	2	1	3	1	3	2	--
Serine	tr	1	0	2	2	1	1	2	2	0	2	2	2	--
Glycine	0	1	1	1	0	0	1	1	1	0	1	2	0	--
Cysteine	0	0	1	1	0	0	0	0	0	0	0	0	0	--
Methionine	0	0	0	1	1	0	0	tr	0	1	0	2	tr	--
Phenylalanine & leucine	0	0	0	1	1	0	0	1	0	1	0	3	1	--
Threonine	0	2	tr	1	1	0	1	1	1	1	1	2	1	--